5/21-29/98

5/21 Purification of 1D3 B1 Monoclonal Antibody by Protein G Chromatography

In order to determine if the antibody produced by our 1D3 hybridoma is clonal or if the prep we isolated on the protein G column on 5/6 was contaminated with 33D1 antibody, we are purifying antibody from the supernatant of a limiting dilution clone of 1D3 - 1D3 B1. This way we will know that the hybridomas are clonal (so that the population of antibody being produced is clonal).

- Followed the procedure on pg. 35 except prior to using the column, I stripped it with 0.1M glycine pH 2.3 (as opposed to the regular pH 2.8 0.1M Glycine) according to the recommendations of Pierce.

Started with 18ml of hybridoma supt. Diluted 1:1 with binding buffer. Ran over equilibrated column over a 5 hour period. Eluted in 1ml 0.1M glycine pH 2.8 - neutralized immediately w/ 25µl of 1M Tris pH 9 / 1ml fraction.

Sample TD λ 280.0
1.5 → Factor 1.000

	Abs	Result mg/ml
1	0.0002	0.0002
FR 1	0.0009	0.0009
FR 2	0.1902	0.1902
→ FR 3	0.6907	0.6907
FR 4	0.2753	0.2753
FR 5	0.1429	0.1429
FR 6	0.0796	0.0796
FR 7	0.0466	0.0466

ε, extinction coefficient for IgG = 1.5

$\varepsilon = \frac{Abs \times \text{path length}}{C}$

$\therefore C = \frac{Abs}{\varepsilon}$

∴ FR 2 = 0.1268 mg/ml
FR 3 = 0.4600
FR 4 = 0.184
FR 5 = 0.095
FR 6 = 0.053
FR 7 = 0.031
Σ 0.950mg/18ml

All are 1ml fractions
0.950mg/18ml = 0.053mg/ml
or 53µg/ml

This is likely mainly

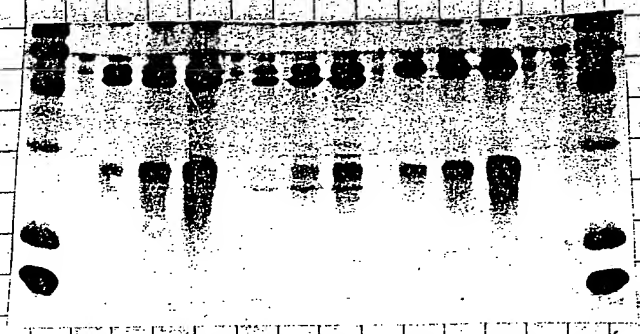
5/22 Silver Stain of Purified 1D3 B1 Antibody

- 15% PAGE gel
- Samples:

Lane	Sample	Quantity Loaded	Estimated Quantity Loaded (based on A280 — 1 µg each protein)
1	High MW markers	1 µl	
2	Blank		
3	Prep of 3/23 - 1D3	2.5 µl	0.675 µg
4	"	5 µl	1.35 µg
5	"	10 µl	2.7 µg
6	Blank		
7	Prep of 5/21 - 1D3 B1	1 µl	0.46 µg
8	"	2.5 µl	1.15 µg
9	"	5 µl	2.3 µg
10	Blank		
11	33D1 Ab	1 µl	0.58 µg
12	"	2 µl	1.16 µg
13	"	4 µl	2.32 µg
14	Blank		
15	Blank		
16	High MW markers	1 µl	1 µg (each protein)

Fraction 3: A280 = 270 µg/ml
Fraction 3: A280 = 460 µg/ml
580 µg/ml

Photo taken on Eagle Eye - Transilluminator



The prep #2 still looks contaminated with 33D1 - Must be bottle - We'll go with it for the bioassay.

Our 2.5 µl sample looks like the 33D1 2 µl sample at 1.16 µg
∴ estimated conc = 1.16 µg / 2.5 µl = 0.464 µg/µl or 0.464 mg/ml
(464 µg/ml)

5/29 BCA Assay to Determine Concentration of "Purified" 1D3-B1 (mAb to STAT3)

The A280's of the protein G column fractions seemed to be overestimated based on the bands I got when I loaded the SDS-PAGE gel. Therefore, I will do a BCA assay to more accurately determine the concentration of the "purified" 1D3 B1 Antibody prep that will be used in biological assays. We're most interested in fraction 3 because it appears to have the most Ab, but I'll throw in fractions 2 & 4 as a reference to compare to the A280 measurements.

- Enhanced protocol = 30 min at 60°C - microtiter plate
- Added 10µl of sample to 200µl of BCA reagent, run in duplicate
- Blank = Tris/Glycine

BSA standard in µg/ml

	1	2	3	4
A	0.744 (1/00)	0.758	0.167 (1/2)	0.164
B	0.758 (600)	0.739	0.097 (1/2)	0.098
C	0.574 (300)	0.530	0.570 (1/4)	0.562
D	0.460 (400)	0.461	0.353 (1/2)	0.378
E	0.557 (500)	0.533	0.135 (1/4)	0.131
F	0.636 (600)	0.608	0.250 (1/4)	0.236
G	0.745 (700)	0.712	0.116 (1/4)	0.121
H	-0.0072 (1/2)	0.001		

Concentration	x̄ Absorbance
100 µg/ml	0.186
200	0.2485
300	0.327
400	0.4605
500	0.555
600	0.622
700	0.730

Regression Line:

$y = 7.4571 \times 10^{-2} + 9.3107 \times 10^{-4} x$

$r^2 = 0.993$

		Abs		BCA conc est.	-vs-	A280 conc est.
Fraction 2	N =	0.1965	→	131 µg/ml	130 µg/ml	127 µg/ml
	1/2 =	0.0975 x 2 = 0.195	→	129 µg/ml		
Fraction 3	N =	0.516	→	528 µg/ml	574 µg/ml	460 µg/ml
	1/2 =	0.2805 x 2 = 0.561	→	522 µg/ml		
	1/4 =	0.133 x 4 = 0.532	→	491 µg/ml		
Fraction 4	N =	0.243	→	181 µg/ml	178 µg/ml	184 µg/ml
	1/2 =	0.1185 x 2 = 0.237	→	174 µg/ml		

7/20/98 – 8/8/98

7/20 ID3 B1-4th Prep ⇒ Activity in TNF Bioassay.

Before we use this prep of ID3 B1 to inject mice in our immunotherapeutic experiments, we need to confirm that it neutralizes STNFR binding to TNF. Therefore, we will re-run the bioassay of 6/5 (pg 63) on this prep, as well as the 5/21 that was used in the 6/5 bioassay.

Protocol - same as that on pg 63

Results:

There was no killing in any of the wells with TNF. In addition, the actinomycin D did not seem to inhibit the growth of the cells such that the staining dropped by what is typical. In fact, some of the wells with actinomycin D showed more staining than with media alone.

So there a problem with the cells, the actinomycin, and/or the TNF.

Ryan will test our batch of actinomycin D versus the batch we got from Mike using the lot of TNF received on 6/5/98. In addition, we will order some more TNF because the previous batch is about gone.

Ryan observed little inhibition of growth by the actinomycin D and no killing by TNF. So this due to the passage # of clone K - we've never seen this before. It is likely not an inherent defect of the cells, because I saw the same inactivity on Clone 39 cells.

8/8 TNF Bioassay of 6/22 + 7/10 Preps of 1D3 B1

We need to confirm the biological activity of 1D3 B1 in neutralizing the binding of TNF to STNFR.

Same assay as on pg. 63
Incubated plate w/ Act and TNF for 24 hours at 37°C

7/10/98 Prep of 1D3 B1 (24 hour assay)

	No Ab 1	No Ab 2	No Ab 3	0.1 µg/ml Ab 4	0.1 µg/ml Ab 5	0.1 µg/ml Ab 6	1 µg/ml Ab 7	1 µg/ml Ab 8	1 µg/ml Ab 9	10 µg/ml Ab 10	10 µg/ml Ab 11	10 µg/ml Ab 12
				cells alone			cells + Act D					
DMEM A	0.005	-0.002	-0.001	[0.344	0.337	0.427]	[0.330	0.353	0.335]	~~0.475~~	~~0.516~~	~~0.574~~
TNF 0 B	0.337	0.320	0.335	0.332	0.345	0.319	0.340	0.330	0.330	0.330	0.306	0.336
6.25 C	0.317	0.298	0.330	0.305	0.304	0.307	0.299	0.307	0.285	0.306	0.307	0.332
12.5 D	0.303	0.321	0.327	0.334	0.296	0.315	0.318	0.309	0.302	0.272	0.290	0.328
25 E	0.297	0.307	0.297	0.288	0.306	0.313	0.280	0.297	0.304	0.297	0.294	0.324
50 F	0.297	0.297	0.304	0.298	0.311	0.301	0.301	0.286	0.273	0.288	0.265	0.298
100 G	0.280	0.290	0.244	0.289	0.285	0.291	0.281	0.298	0.285	0.280	0.262	0.317
H	~~0.774~~	~~0.517~~	~~0.530~~	~~0.770~~	~~0.518~~	~~0.474~~	~~0.505~~	~~0.456~~	~~0.479~~	~~0.453~~	~~0.544~~	~~0.577~~

* Again, there is no difference between "cells alone" and "cells + Actinomycin D". Either the cells are resistant to Actinomycin, or the Actinomycin has gone bad. Decided to incubate the plate an additional 12 hours to determine if the Actinomycin D is active.

[redacted]

6/22/98 Prep of 1D3 B1 (36 hour assay)

	No Ab 1	No Ab 2	No Ab 3	0.1 µg/ml Ab 4	0.1 µg/ml Ab 5	0.1 µg/ml Ab 6	1 µg/ml Ab 7	1 µg/ml Ab 8	1 µg/ml Ab 9	10 µg/ml Ab 10	10 µg/ml Ab 11	10 µg/ml Ab 12
				cells alone			cells + Act					
DMEM A	0.003	-0.004	0.000	[0.530	0.548	0.530]	[0.230	0.226	0.231]	~~0.454~~	~~0.537~~	~~0.414~~
TNF 0 B	0.242	0.235	0.245	0.228	0.222	0.234	0.234	0.229	0.235	0.224	0.244	0.230
6.25 C	0.187	0.214	0.218	0.207	0.207	0.201	0.209	0.205	0.216	0.220	0.214	0.241
12.5 D	0.217	0.205	0.213	0.200	0.213	0.212	0.207	0.216	0.207	0.216	0.206	0.188
25 E	0.193	0.196	0.207	0.205	0.195	0.207	0.200	0.207	0.196	0.209	0.207	0.202
50 F	0.183	0.177	0.190	0.184	0.195	0.204	0.203	0.200	0.191	0.218	0.193	0.190
100 G	0.168	0.172	0.180	0.175	0.170	0.182	0.179	0.187	0.177	0.189	0.197	0.194
H	~~0.544~~	~~0.548~~	~~0.414~~	~~0.481~~	~~0.474~~	~~0.475~~	~~0.508~~	~~0.527~~	~~0.540~~	~~0.551~~	~~0.509~~	~~0.530~~

The Actinomycin D does appear to be active: cells alone = 0.536
Cells + Act = 0.229 (43% inhibition). We are not, however, observing much killing with TNF:

no antibody data		10 µg/ml Ab data	
0	0.241 = –	0	2.33
6.25	0.206 = 15%	6.25	0.226 – 3%
12.5	0.210 = 13%	12.5	0.203 – 13%
25	0.199 = 17%	25	0.203 – 13%
50	0.182 = 24%	50	0.200 – 14%
100	0.173 = 28%	100	0.193 – 17%

Maybe we should extend the incubation period – go back to original protocol for plate + maybe do a three day assay w/ [illegible] samples

11/23/98

DISSERTATION

THE ROLE OF SOLUBLE TUMOR NECROSIS FACTOR RECEPTOR TYPE I IN TUMOR SURVIVAL

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